

Photoreduction of Sulfur Dioxide by Spinach Leaves and Isolated Spinach Chloroplasts

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ABSTRACT

Labeled sulfur dioxide was found to be extensively absorbed by spinach (*Spinacea oleracea* L.) leaves. Labeled sulfides detected in leaf blades following fumigations with sulfur dioxide in light indicated that photoreduction of sulfur dioxide had occurred. Measurable proportions of this labeled sulfur was localized within the chloroplast fraction. Suspensions of isolated chloroplasts supplied with labeled sulfur dioxide contained labeled sulfides following a 30-minute illumination period in water-cooled reaction vessels. With reference to recent studies of the chloroplast sulfur reduction pathway, probable points of entry for sulfur dioxide and the subsequent release of hydrogen sulfide are discussed.

Sulfur dioxide is a major atmospheric contaminant and phytotoxicant resulting from the combustion of sulfur-containing fossil fuels. The phyto-toxic behavior of the gas has received considerable attention (6, 8, 18). Foliar absorption studies have revealed that SO₂ is extensively absorbed by leaves when stomates are open (3, 6), suggesting that vegetation may act as an important "sink" for atmospheric pollutants (7).

Cormis (5) demonstrated that plants exposed to SO₂ released measurable quantities of H₂S into the surrounding atmosphere. The phenomenon was shown to result from a photoreductive process since no H₂S was detected during fumigations with SO₂ in darkness. The present investigation examines certain aspects of the foliar absorption of SO₂, its cytoplasmic distribution, and a possible mechanism allowing formation of hydrogen sulfide.

MATERIALS AND METHODS

Chamber Fumigations. Six- to eight-week-old spinach plants (*Spinacea oleracea* L.), grown in a controlled-environment cabinet, were exposed to ³⁵SO₂ in a glass fumigation chamber (17). The chamber consisted of an 18-liter bell jar, the basal flange of which was clamped to form an airtight seal with an inert-surfaced supporting platform. A squirrel-cage fan blade located within the chamber was used to circulate air within the closed system. Baffles situated in order to direct the air flow pattern also served as a supporting platform for a single pot of plants.

All procedures were performed within the confines of a laboratory hood. Sulfur dioxide containing ³⁵SO₂ was generated from the reaction of H₂³⁵SO₄ (International Chemical and Nuclear Corp., Irving, Calif.) with thin copper "turnings." The pressure generated by externally applied heat and the reaction carried the

evolved gas and confined air through an impinger containing concentrated H₂SO₄ to remove water vapor and then into a 30-liter Mylar gas bag (Calibrated Instruments Co., Ardsley, N.Y.) contained in a second bell jar. When the SO₂ generation was completed, this supply system was isolated by turning a three-way valve attached to the inlet of the Mylar gas bag which served as a gas reservoir. A measured quantity of laboratory air was then added to the bag to dilute the gaseous mixture to the desired SO₂ concentration for introduction into the chamber. Withdrawal of a measured volume of the radioactive gas mixture was accomplished by a 50-ml gastight syringe attached to the gas line leading from the reservoir to the fumigation chamber. Measured aliquots could either be expelled into the fumigation chamber, or collected in midjet impingers containing 0.03 N H₂O₂ solutions for analysis (10).

Illumination during the fumigations was provided by a bank of five 150-w reflector flood lamps yielding a light intensity of 1200 ft-c at the position of the leaves within the chamber. Plants were illuminated in position for 20 min before lowering the bell jar over them in preparation for exposure to labeled SO₂. The chamber temperature during fumigations was 29 ± 2 C.

Immediately after clamping the chamber in place, a measured volume of the labeled SO₂ gas mixture sufficient to produce an atmosphere containing 5 μl/l SO₂ was introduced into the chamber. To compensate for observed decreases in SO₂ concentration due to absorption by leaves and inner chamber surfaces, a second SO₂ gas sample of like volume and composition was introduced into the chamber 10 min after the first addition. After a 30-min exposure time, the air in the chamber was flushed through impingers containing aqueous H₂O₂ and replaced simultaneously with inflowing charcoal-filtered air.

Measurement of Radioactivity. Measurement of ³⁵SO₂ was accomplished by drawing gas samples through an impinger containing 10 ml of 0.03 N H₂O₂. A 0.1-ml aliquot of the absorbing solution was then placed in a scintillation vial with scintillation liquid prepared according to Wang and Willis (21). Leaf samples were digested according to the procedure of Mahin and Lofberg (12), prior to scintillation counting. Radioactivity was determined, using a Beckman Model LS-100 liquid scintillation counter.

Determination of ³⁵SO₂ in Chloroplasts. Following the fumigation period, 10 g of leaves were harvested and washed in distilled H₂O to remove labeled sulfur absorbed on the outer leaf surfaces. Using these leaf samples, intact chloroplasts were isolated from major portions of other cytoplasmic fractions, using sucrose gradient methods described by Leech (11). Chloroplasts prepared in this manner are hereafter designated "isolated chloroplasts." Chl content was determined according to Arnon (1).

Estimations of Photoreduction of SO₂ by Spinach Leaves. "Acid-volatile" H₂S extractions from leaf samples of spinach plants exposed to SO₂ were performed according to the method of Shevyokova (16). The H₂S was carried from the acidified leaf

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samples in a stream of N_2 and collected in 10 ml of a $Cd(OH)_2$ solution. Spectrophotometric sulfide analysis was performed according to the method described by Katz (10), using a Hitachi Perkin-Elmer Model 139 UV Vis spectrophotometer.

Estimations of Photoreduction of SO_2 by Spinach Chloroplasts. Suspensions of photosynthetically active intact chloroplasts were prepared from freshly harvested spinach leaves by the method of Kalberer *et al.* (9). The reaction mixture contained (in μ moles) the following in a total volume of 3 ml: tris-HCl (pH 7.8), 100; mannitol, 400; Na-isoascorbate, 2.5; $MgCl_2$, 40; ADP, 5; K_2HPO_4 , 10; EDTA, 2; and chloroplasts representing 300 μ g Chl.

The reaction was conducted in a round bottom flask equipped with a gas dispersion tube. The flask was attached to a cold-finger condenser by a ground glass fitting and immersed in a water bath maintained at 15 C. A 50-ml gastight syringe, equipped with a three-way valve located between the reaction flask and a Mylar gas bag reservoir, was used to withdraw and transfer measured volumes of labeled SO_2 (about 110,000 cpm) from the bag into the chloroplast suspension held in the reaction flask. The total quantity of SO_2 , labeled and nonlabeled, present in each gas sample ranged from 0.8 to 1.2 μ moles.

The photoreactions were allowed to proceed at 20 C for 30 min in the light (3000 ft-c) under N_2 , and were then stopped by addition of 1.3 ml of 20% (w/v) trichloroacetic acid. All acid volatile SO_2 and H_2S was then trapped by a 30-min ebullition with N_2 through an H_2S -absorbing solution containing 0.1 M $CdSO_4$ and 1% (w/v) lactic acid (20). Approximately 1.5 mmoles of Na_2S were added to the absorbing solution and the resultant CdS precipitate collected for analysis of labeled sulfide by liquid scintillation methods described previously. Data obtained were expressed as cpm sulfide/mg Chl as an index of H_2S production by the suspended chloroplasts.

RESULTS

Absorption, Distribution, and Photoreduction of SO_2 by Spinach Leaves. The quantity of $^{35}SO_2$ absorbed and retained within spinach leaves is shown in Table I. In two of the experimental runs (Nos. 3 and 4), where total fresh weight of the exposed

portions of the plants had been measured, calculations based on radioactivity in leaf blade samples were made to determine the amount of SO_2 absorbed by the plants relative to the total SO_2 introduced into the chamber atmosphere. These indicated that 17.6 and 18.7% of the total SO_2 introduced into the chamber was absorbed by the spinach foliage in experiment Nos. 3 and 4, respectively.

Measurable quantities of labeled sulfur were detected in chloroplasts isolated from fumigated leaves (Table I). Mean Chl content of 10 spinach leaf blade samples was 1.83 mg Chl/g fresh weight, as determined by acetone extraction and spectrophotometric analysis (1). Using this mean Chl content as a "conversion factor," the percentage of labeled sulfur absorbed that was actually present with the isolated chloroplast fraction may be calculated. Considering, for example, experiment No. 1, out of a total of 10,315 cpm/g fresh weight, approximately 83 cpm/mg Chl \times 1.83 mg Chl/g fresh weight, or 154 cpm (1.5%) of the absorbed label was present in the chloroplast fraction. Similar calculations for all fumigations show that from 1.3 to 3.2% of the absorbed sulfur is associated with isolated chloroplasts.

Acid volatile sulfides were measured in leaf blade samples of both SO_2 -fumigated and SO_2 -nonfumigated plants to determine whether increased foliar sulfide levels result from exposure to SO_2 . Acid-volatile sulfide content of nonfumigated spinach leaf samples ranged from 3.6 to 7.6 μ g/g fresh weight (Table II). The acid-volatile sulfide content of spinach leaves exposed to SO_2 ranged from 6.2 to 9.8 μ g/g fresh weight.

Photoreduction of SO_2 by Spinach Chloroplasts. The data presented thus far have shown that sulfur becomes associated with the chloroplasts within leaves of illuminated plants exposed to SO_2 , and measurable quantities of the absorbed sulfur are reduced to S^{2-} in light. The light requirement (5) suggests that sulfide is the product of the photoreduction demonstrated to occur within the chloroplast (14, 15). This hypothesis was examined by exposing suspensions of isolated spinach chloroplasts to $^{35}SO_2$ and measuring the resultant $^{35}S^{2-}$ produced.

Chloroplast suspensions displayed the following average rates of $^{35}S^{2-}$ production in separate experimental runs: 2133, 4086, 1841, and 1866 cpm S^{2-} /mg Chl, based on acid-volatile sulfide yield. Labeled sulfide was not detected when osmotically rup-

Table I. Sulfur-35 Content of Spinach Leaf Blades and Intact Chloroplasts Isolated from Leaves following a 30-min Exposure of Spinach Plants to $^{35}SO_2$ in a Lighted Fumigation Chamber

Experiment Number ¹	$^{35}SO_2$ Introduced into Chamber ²	Sulfur-35 Content ³			
		Leaf Blades	Isolated Chloroplasts		
	cpm	cpm/g fr wt	cpm/mg Chl	cpm (corrected) ⁴	% ⁵
1	782,000	10,315	84	154	1.5
2	660,000	5,784	100	183	3.2
3	822,000	7,668	128	234	3.0
4	824,000	8,121	110	201	2.5
5	1,243,000	16,500	116	212	1.3

¹Corresponding Experiment Numbers in Tables I and II identify data obtained from the same fumigation treatment.

²Maximum SO_2 concentrations of 10 μ l/l in experiments 1 through 4, and 15.5 μ l/l in #5.

³Each value represents the calculated mean based on counts of replicate samples.

⁴Each gram (fr wt) of tissue contained 1.83 mg Chl (Materials and Methods). Therefore, cpm/mg Chl \times 1.83 represents cpm (corrected) contributed by the chloroplast fraction of each gram of tissue.

⁵Percentage of the absorbed label that was recovered in isolated chloroplasts, or cpm (corrected)/(total cpm/g fr wt) \times 100%.

Table II. Quantitative Comparison of Sulfides in Leaf Blade Samples of SO_2 -Fumigated and Nonfumigated Spinach Plants

Experiment Number	Sulfides, Acid Volatile ¹	
	Nonfumigated	Fumigated
	$\mu\text{g/g fr wt}$	
1	3.6	8.8
2	4.2	6.2
3	7.6	9.8
4	5.4	7.7
5	6.2	6.6
Mean	5.4	7.8

¹ Acid volatile sulfide released by the method of Shevyakova (16).

² Leaf blade samples were taken from plants exposed to SO_2 in experiments 1 through 5. See Table I and text for explanation.

³ Comparison of $\mu\text{g S}^{2-}/\text{g fr wt}$ released from fumigated versus nonfumigated leaf blade samples using "Student's" t -distribution shows a significant difference, with $P < 0.01$.

tured chloroplasts plus 0.03 μmole of spinach ferredoxin and 3 μmoles of oxidized glutathione were substituted for intact chloroplasts. Nor was the label recovered when the reaction mixture was acidified immediately following addition of $^{35}\text{SO}_2$.

DISCUSSION

The results of this investigation suggest that SO_2 is absorbed by leaves in sufficient quantities to account for the metabolic disturbances and phytotoxic reactions which normally result (6, 9, 18). Bennett and Hill (3) have measured the rates of absorption of SO_2 by standardized plant canopies. The high uptake rates of SO_2 which they observed were attributed to the great solubility of the gas in the aqueous media bathing the cells and to the plant's capacity to deplete the resulting solute concentration of these aqueous media through translocation and metabolic activity.

Cormis (5) detected H_2S emission into the surrounding atmosphere within 30 min following initial exposure of plants to SO_2 . This would suggest that sulfur anions, formed following absorption of SO_2 by leaves, soon reach the chloroplast, the demonstrated site of sulfur reduction (14, 15). The resulting H_2S formed during the photoreduction process is apparently released from the chloroplast and then from the cytoplasm of the mesophyll cells into the ambient air with similar rapidity. Therefore, the sulfur content of chloroplasts measured following a 30-min fumigation (Table I) may represent a dynamic equilibrium level of ^{35}S rather than a simple accumulation.

Chloroplast preparations similar to those in which we have observed photoreduction of SO_2 , were also capable of reducing either SO_4^{2-} or SO_3^{2-} to S^{2-} (15, 20). Dissolved SO_2 converts readily to HSO_3^- and SO_3^{2-} , and is oxidized only slowly to SO_4^{2-} (2, 13, 18). It is likely that gassing the buffered chloroplast suspension with SO_2 was, in effect, analogous to direct introduction of sulfite ions. The question thus emerges as to whether the observed reduction of SO_2 to sulfides can be accounted for by the normal pathway of sulfate reduction in chloroplasts as theorized by Schiff and Hodson (14). These authors suggest that

while the intermediates in the pathway between SO_4^{2-} and S^{2-} appear to be bound to carrier proteins, an equilibrium relationship may exist between bound and free forms of SO_3^{2-} and S^{2-} . If this is true, sulfur from SO_2 may enter the reductive pathway as either SO_4^{2-} or SO_3^{2-} . Sulfide, in turn, may either enter amino acid synthesis or be released from the carrier. Protonation of the free S^{2-} within the chloroplast would yield H_2S which may escape from the leaves.

More recently, Tamura and Itoh (19) have observed sulfite photoreduction in a spinach grana-ferredoxin system supplemented with a thermolabile sulfite reductase from the leaf extract. Their data suggest that sulfite functions as a ferredoxin-linked terminal electron acceptor in a photoreduction similar to that of nitrite or pyridine nucleotide. This system could easily account for photoreduction of foliar-absorbed SO_2 to sulfide via the sulfite ion.

Results of the present investigations suggest that sulfur reduction mechanisms which function in spinach chloroplasts could account for production and emission of H_2S from illuminated plants exposed to SO_2 . In addition, the photoreductive activity observed in the present study may be related to phytotoxic effects of SO_2 and should be considered along with other mechanisms currently being proposed to account for these phytotoxic effects (4, 6, 17, 18).

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